BIOSORPTION OF COPPER IONS BY SUBMERGED MYCELIUM OF SCHIZOPHYLLUM COMMUNE

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Abstract
Biosorption of copper ions by the submerged mycelium of wood-rotting fungus Schizophyllum commune and the components of its cell wall were studied. Chitin-glucan complex (CGC I) exhibited the highest sorption capacity (0.3 mMol/g). The biosorbent CGC I was described in terms of microstructure, the effects of conditions on the Cu$^{2+}$ sorption and sorption dynamics and selectivity. CGC I was suggested as a potential biosorbent for heavy metal removal from drinking water.

Key words: Biosorption, chitin-glucan complex, Schizophyllum commune, copper, heavy metals

Introduction
Many methods are being used to remove heavy metals from water nowadays (Fenglian, Wang, 2011). In the last decades, seeking new, economically effective technologies for heavy metal removal has been focused on biosorption. This very perspective alternative is based on a non-specific physio-chemical interaction between a metal ion and an organic compound. Major advantages of biosorption are low cost, high efficiency at low metal concentrations and possibility of metal regeneration (Alluri et al., 2007).

Often studied biosorbents are bacteria, algae and agriculture and food industry waste. Multiple commercial biosorbents based on a biomass of these organisms or compounds for waste water remediation have been produced (Michalak, Chojnacka, Witek-Krowiak, 2013). In the recent years, attention has been paid to molds, e.g. Aspergillus niger, Penicillium lanosa, Rhizopus nigricans, etc. whose mycelium is a waste of biotechnological productions. Sorption of heavy metals by higher fungi is very little examined.

Schizophyllum commune is a wood-rotting fungus from the Agaricales order which grows on all continents except Antarctica. The fungus is not poisonous; it is considered as nonedible for its toughness, though. However, in some regions of the world it is commonly consumed (Mexico) (Ruán-Soto, Garibay-Ortijel, Cifuentes, 2006). S. commune has a biotechnological significance because when cultivated in a liquid medium, it produces an extracellular polysaccharide β-glucan schizophyllan. This polysaccharide is used for cosmetic purposes because of its ability to hydrate skin and improve its structure. During the production of the above mentioned polysaccharide in Contipro Group s.r.o., S. commune is cultivated in submerged culture and schizophyllan is obtained from the filtrate while the mycelium is a by-product.

The aim of the study was to find out whether the S. commune mycelium could be used as a source for production of an efficient biosorbent exploitable for removing heavy metals from water.

Methods and material
Fungus inoculation and cultivation
S. commune was stored at the temperature of +4°C on a slant agar with PDA (potato dextrose agar). The culture from the slant agar was used for inoculation of Petri dishes with PDA. Then, the Petri dishes were cultivated in an incubator at the temperature of +29°C for 7 days. The Petri dishes were used to inoculate 1000 mL Erlenmeyer flasks with 500 mL of liquid nutrition medium. The medium contained (in g/L): 35 – sucrose, 4 – yeast extract, 2.5 - Na$_2$HPO$_4$×12H$_2$O, 0.5 - MgSO$_4$×7 H$_2$O; pH of the medium was altered to 6.0 using HCl. The Erlenmeyer flasks were cultivated on a shaker at
29°C, 200 rpm for 5 days and afterwards used for inoculation of bioreactor Techfors-S with operational volume of 20 L (Infors HT, Switzerland). The parameters of the bioreactor: aeration 2 vvm, agitation 250 rpm, 29°C. Cultivation lasted for 7 days.

When the cultivation was terminated, the mycelium was obtained from the nutrition medium by filtration through a synthetic fabric. The mycelium was then washed of the medium by three times of the volume of demineralized water and used for further experiments.

Biosorbents preparation

Washed and milled mycelium was used as a raw material to prepare the biosorbents. Submerged *S. commune* mycelium (100 g) was dispersed in 3 L demineralized water and the suspension was homogenized for 15 minutes in a colloid mill IKA Magic LAB mill with MK-module for colloid milling at the rotational speed 16 000 rpm.

The sample “mycelium” was prepared as follows. Washed and milled mycelium was centrifuged at RCF 10 000 x g, the supernatant was removed and the sediment (mycelium) was resuspended in 3 L demineralized water and homogenized. This process was repeated until the removed supernatant reached zero absorbance for wavelengths 250 – 700 nm (measured by UV-1800 Shimadzu Spectrophotometer with demineralized water as control). Completely washed mycelium was frozen and lyophilized.

The sample “digested mycelium” was prepared as follows. Washed and milled mycelium was centrifuged (RCF 10 000 x g; 20 min), the supernatant was removed and the sediment (mycelium) was resuspended in NaOH aqueous solution (1 g mycelium / 50 mL water with 0.5 g NaOH). The mycelium was incubated for 1 hour at constant stirring and temperature of 80°C. The mixture was then centrifuged, the supernatant was removed and the sediment was resuspended in demineralized water by Ultra-TURRAX T25 Digital (IKA, Germany) and centrifuged again. This process was repeated until the pH of the supernatant dropped below 10. Afterwards, the sample was frozen and lyophilized.

The sample “cell wall” was prepared as follows. Washed and milled mycelium was centrifuged (RCF 10 000 x g; 20 min), the supernatant was removed and the sediment (mycelium) was frozen at -80°C. Then, the sample was triturated in a ceramic bowl and suspended in demineralized water. This suspension was then exposed to ultrasound for 60 minutes in order to break down the cell walls. Then, the suspension was centrifuged, the supernatant was removed and the sediment was resuspended in demineralized water by Ultra-TURRAX T25 Digital (IKA, Germany) and centrifuged again. This process was repeated until the removed supernatant reached zero absorbance for wavelengths 250 – 700 nm (measured by UV-1800 Shimadzu Spectrophotometer with demineralized water as control). Finally, the sample was frozen and lyophilized.

The sample “chitin-glucan complex I” (hereinafter “CGC I”) was prepared as follows. Washed and milled mycelium was centrifuged (RCF 10 000 x g; 20 min), the supernatant was removed and the sediment (mycelium) was resuspended in NaOH aqueous solution (1 g mycelium / 50 mL water with 12.5 g NaOH), homogenized by Ultra-TURRAX T25 Digital (IKA, Germany), heated to 90°C and incubated at this temperature for 3 hours at constant stirring. The mixture was then centrifuged, the supernatant was removed and the sediment was resuspended in demineralized water by Ultra-TURRAX T25 Digital (IKA, Germany) and centrifuged again. This process was repeated until the pH of the supernatant dropped below 10. The sediment (chitin-glucan complex I) was then frozen and lyophilized.

The sample “chitin-glucan complex II” (hereinafter “CGC II”) was prepared using the same method as the sample “chitin-glucan complex I”. The difference was that after the mycelium was altered with the sodium hydroxide and the pH of the supernatant dropped below 10, the sediment was mixed with 0.25 M lactic acid...
(1 g mycelium / 10 mL 0.25 M lactic acid), resuspended by Ultra-TURRAX T25 Digital (IKA, Germany) and incubated for 2 hours at 50°C. The resulting CGC II was centrifuged, the supernatant was removed and the sediment was resuspended in 300 mL demineralized water by Ultra-TURRAX T25 Digital (IKA, Germany) and centrifuged again. This process was repeated until the removed supernatant reached conductivity below 75 μS/cm. CGC II was frozen and lyophilized.

Analytical methods

0.5 g of the sorbent was put into a centrifuge tube (50 mL). Using a pipette, 30 mL 1% CuSO$_4$ × 5 H$_2$O was added, the pH of the solution was adjusted to 4.4 and the content of the tube was homogenized with a glass stick which was washed with demineralized water after removing from the cuvette. The cuvette was incubated at the room temperature at a shaker for 30 minutes at 200 rpm. Then, the content of the cuvette was centrifuged for 10 minutes at 10 000 x G. After removing the supernatant, 30 mL demineralized water was added to the cuvette and the sediment was homogenized by Ultra-TURRAX T25 Digital (IKA, Germany). The cuvette was centrifuged again at the above described conditions. The supernatant was removed again and the sediment was resuspended in 20 mL 0.1 M sulfuric acid. The content of the cuvette was quantitatively transferred into a titration flask. 4 mL 30% potassium iodide solution and 2 ml 0.5% starch solution were added by a pipette to the flask. The content of the flask was immediately titrated by 0.1 M sodium thiosulfate solution to a light yellow to milky coloration. The consumption of sodium thiosulfate was measured with the accuracy of 50 μL. From the titrant consumption, the sorption capacity (SC) was calculated in mMol Cu$^{2+}$ per 1 gram of sorbent.

$$SC = \frac{32.44 \cdot f \cdot a}{m \cdot S - (16 \cdot f \cdot a)}$$

- $a$ - titrant consumption [mL]
- $f$ - titrant factor, 1
- $m$ - sample weight [mg]
- $S$ - sample dry matter [%]

In order to examine the effect of pH on the sorption capacity, the pH of the 1% CuSO$_4$ × 5 H$_2$O solution was altered by NaOH or H$_2$SO$_4$ to values 1.0, 2.0, 3.0, 4.0 and 5.0. The further experiments were performed as stated above.

Studying the dynamics of the sorption proceeded in the same way as examining the sorption capacity except that the cultivation time was changed to 2.5, 5 and 15 minutes. Filtration through a synthetic fabric was used after the sorbent (CGC I) was separated from the copper solution.

To determine the effect of the Cu$^{2+}$ ions concentration in the solution on the CGC I sorption capacity, the CuSO$_4$ × 5 H$_2$O concentration was changed to 1%; 0.1%; 0.05%; 0.025% a 0.0125%.

To investigate the sorption metal selectivity, a solution of the following salts was used CaCl$_2$, ZnCl$_2$, MgCl$_2$ × 6 H$_2$O, NaCl, CuSO$_4$ × 5 H$_2$O, C$_4$H$_4$CdO$_4$ × 2 H$_2$O, CoCl$_2$ × 6 H$_2$O. The final concentration of the metal ions (Ca$^{2+}$, Zn$^{2+}$, Mg$^{2+}$, Na$^+$, Cd$^{2+}$, Co$^{2+}$) was 0.004 mol/L. The concentration of the individual metals in the desorbate was determined by ICP- OES Radial 725 (Agilent Technologies, USA).

All of the samples were scanned by a scanning electron microscope ULTRA PLUS (Carl ZEISS). The parameters of the microscope were: acceleration voltage = 1.3 kV, operational distance 4.3-4.5 mm, current = 31 pA, high vacuum (1x10$^{-4}$ Pa), room temperature. A 15 nm layer of gold particles was applied to every sample by SC7620 Mini Sputter Coater (Quorum Technologies, UK).
Results and Discussion
The growth of *S. commune* and production of mycelium in liquid medium were studied during the cultivation in a bioreactor with 20 L operational volume. The fungus reached the stationary growth phase within 120 hours after inoculation when the biomass yield reached 22.6 g/L of medium (Fig. 1.)

![Mycelium concentration variation during the *S. commune* growth in a bioreactor](image)

Based on the results obtained, *S. commune* may be classified amongst fungi with high growth rate. For comparison, the micromycetes from the Zygomaticum division produced 4 – 8 g/L mycelium within 74 – 96 hours of cultivation (Su Ching Tan et al. 1996). The higher fungi from the Basidiomycetes division produced 3.5 – 6.8 g/L mycelium within 21 days of submerged cultivation (Mario et al. 2008).

The ability of the *S. commune* mycelium to absorb copper ions from an aqueous solution was determined. The sorption capacity of the mycelium was compared to the sorption capacity of the active carbon which is commonly used as a sorbent in filtration systems (Dean, Bosquil, Lanouette, 1972) and to an enterosorbent Smecta® that contains aluminum silicates and magnesium silicates as its active components. The experiment showed that the mycelium absorbs twice more copper ions than the active carbon but its sorption capacity is five times lower than the capacity of Smecta® (Tab. 1.). Compared to other biosorbents on fungus mycelium basis, *S. commune* exhibits an average sorption capacity. The sorption capacity of the fungi *Auricularia polytrichia* and *Saccharomyces cerevisiae* mycelium reaches 0.03 mMol/g and 0.02 mMol/g, respectively (Galli et al., 2003; Huang, Huang, Morehart, 1990). The value of the sorption capacity of the fungus *Aspergillus niger* varies from 0.02 to 0.45 mMol/g (Kapoor, Viraraghavan, Cullimore, 1999; Dursun, 2006; Mukhopadhyay, Noronha, Suraishkumar, 2007).

Tab. 1. Sorption of copper ions by *S. commune* mycelium and commercial sorbents

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sorption capacity, mMol Cu$^{2+}$/g sample</th>
</tr>
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<tbody>
<tr>
<td>mycelium <em>S. commune</em></td>
<td>0.07± 0.02</td>
</tr>
<tr>
<td>active carbon</td>
<td>0.03± 0.01</td>
</tr>
<tr>
<td>Smecta®</td>
<td>0.38± 0.07</td>
</tr>
</tbody>
</table>
A fungus mycelium is mostly composed of polysaccharides which are located mainly in the cell wall where they make up for over 80% of its weight. The *S. commune* cell wall polysaccharides may be divided to water soluble and water insoluble (Sietsma, Wessels, 1977). Water soluble polysaccharide is composed mainly of schizophyllan (extracellular β-1,3/1,6-glucan which is located on the outer surface of the cell wall). Water insoluble polysaccharides may be divided based on their solubility in an alkali environment. Polysaccharides soluble in alkali solution are located mostly on the outer surface of the cell wall in a form of a micro-crystal layer and are composed of glucose monomers which are bound together by an α-1,3-bond. A part of the cell wall which is not soluble in an alkali environment is a complex structure that is composed of β-1,3- and β-1,6-branched glucose polymers covalently bound to chitin (chitin-glucan complex) (Sietsma, Wessels, 1979). Different glycopolymers of the mycelium have various affinity to copper ions. By successive removal of the individual fractions of the cell wall polysaccharides, it is possible to find the fraction with the highest sorption capacity.

By successive mycelium extraction with aqueous solutions of NaOH and lactic acid, a set of biosorbents that were composed of different mycelium fractions was prepared: cell wall, digested mycelium, chitin-glucan complex I (CGC I) and chitin-glucan complex II (CGC II). The yield of the individual fractions is shown in Table 2.

**Tab. 2.** The yield of the individual *S. commune* mycelium fractions

<table>
<thead>
<tr>
<th>Mycelium fraction</th>
<th>yield, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>mycelium</td>
<td>100</td>
</tr>
<tr>
<td>cell wall*</td>
<td>-</td>
</tr>
<tr>
<td>digested mycelium</td>
<td>71,8 ± 1,1</td>
</tr>
<tr>
<td>CGC I</td>
<td>28,2 ± 2,0</td>
</tr>
<tr>
<td>CGC II</td>
<td>27,3 ± 3,2</td>
</tr>
</tbody>
</table>

*the value unknown for high loss during sample preparation

The results of the comparison of the individual *S. commune* mycelium fractions’ sorption capacity showed that removing the cytoplasm (fraction: cell wall) does not significantly increase the sorption capacity of the mycelium (Tab. 3.). Removing the water soluble polysaccharides, lipids and proteins from the surface of the cell wall (digested mycelium) had no effect on the sorption capacity. On the other hand, the sorption capacity increased 4 times after removing the polysaccharide soluble in an alkali environment (CGC I). CGC I absorbed 10 times more Cu$^{2+}$ ions than the active carbon and only 1.3 less than Smecta®. Further depolymerization and chitin-glucan complex extraction (CGC II) had negative effects on the copper sorption.

**Tab. 3.** Cu$^{2+}$ sorption by the *S. commune* mycelium components

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sorption capacity, mMol Cu$^{2+}$/g sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>cell wall</td>
<td>0,08 ± 0,01</td>
</tr>
<tr>
<td>digested mycelium</td>
<td>0,07 ± 0,02</td>
</tr>
<tr>
<td>CGC I</td>
<td>0,30 ± 0,06</td>
</tr>
<tr>
<td>CGC II</td>
<td>0,12 ± 0,03</td>
</tr>
</tbody>
</table>

After removing the alkali soluble α-glycans, the functional groups of chitin which has very high sorption ability were uncovered which may explain the higher sorption capacity of CGC I compared to the native mycelium. Moreover, the surface area of the sorbent was enlarged by an alkali extraction which is well visible in the photographs from the electron microscope (Fig. 2). Decline of the sorption capacity of CGC
II may be connected to an excessive chitin-glucan complex degradation and chitosan wash out which probably takes place in the process of the acid mycelium extraction.

**Fig. 2.** Photographs of the individual *S. commune* mycelium fractions obtained by a scanning electron microscope: A - mycelium (magnification 1500x); B - cell wall (magnification 2000x); C - digested mycelium (magnification 3500x); C - CGC I (magnification 4000x); D - CGC II (magnification 1500x).
The effect of pH on copper ion sorption was tested for the superior sorbent CGC I. The results suggest that CGC I may be used as a sorbent in the range of pH from 2.0 to 5.0 while the maximum sorption capacity was measured at the pH 5.0 (Fig. 3). Higher pH values were not studied during the experiment because of water insoluble copper hydroxide precipitation.

![Graph showing pH effect on copper ion sorption by CGC I](image)

**Fig. 3.** The effect of pH on the copper ion sorption by CGC I

Studying the dynamics of the copper ion sorption by CGC I showed that the metal ions bind to the biosorbent within the first minutes. After that, the Cu$^{2+}$ concentration remains in a dynamic equilibrium (Fig. 4). Such a rapid sorption equilibrium attainment is characteristic for biosorbents on fibrous fungi mycelium basis (Galli et al. 2003, Ali 2013) and distinguishes them from the biosorbents prepared from agricultural or food industry wastes which may reach their sorption capacity in over than 60 minutes (Bulut, Tez, 2007; Homagaia, Ghimire, Inoue, 2010; Saeed, Iqbal, Akhtar, 2005).

![Graph showing copper ion sorption by CGC I dependent on time](image)

**Fig. 4.** The copper ion sorption by CGC I dependent on time

One of the major advantages of using CGC I is its efficiency at low metal ion concentrations. The study of the effect of the ion concentration on the sorption capacity revealed that CGC I is already able to absorb copper at concentration of 32 mg/mL. Most of the current heavy metal removal methods fail at such low concentration (Das, Vimala, Karthika, 2008). The CGC I sorption capacity increases with higher Cu$^{2+}$ concentration (Fig. 5.).
The sorption in all of the previous experiments took place in a solution containing ions of only one metal. In the case of sorption in an equimolar solution, CGC I absorbs heavy metals selectively in the following order: Cu > Cd ≈ Zn ≈ Co, while the Na⁺, Mg²⁺ and Ca²⁺ ions remain unabsorbed (Tab. 4.).

<table>
<thead>
<tr>
<th>Ion</th>
<th>Control*</th>
<th>Sample**</th>
<th>ΔμMol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu²⁺</td>
<td>0,19</td>
<td>858,49</td>
<td>858,30</td>
</tr>
<tr>
<td>Cd²⁺</td>
<td>0</td>
<td>201,07</td>
<td>201,07</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>0</td>
<td>188,46</td>
<td>188,46</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>22,02</td>
<td>211,01</td>
<td>189,00</td>
</tr>
<tr>
<td>Na⁺</td>
<td>63,48</td>
<td>60,43</td>
<td>-3,04</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>225,19</td>
<td>261,85</td>
<td>36,65</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>198,85</td>
<td>201,64</td>
<td>2,79</td>
</tr>
</tbody>
</table>

**Conclusion**

The results show that CGC I may be used as a sorbent for a fast and efficient removal of low heavy metal ion concentrations from aqueous solutions. Because the biosorbent is produced from mycelium of a nonpoisonous higher fungi *S. commune*, the risk of mycotoxins contamination is eliminated; thus, CGC I may be used for drinking water remediation. It was proved that a 3 minute treatment of water which contains 1.8 mg/L Cu²⁺ with 4 g/L CGC I is sufficient to remove more than 83% Cu²⁺ and bring the water within the accordance with WHO Drinking Water Standards (less than 1 mg/mL Cu²⁺).

The waste product of the schizophyllan polysaccharide cosmetic production, the *S. commune* submerged mycelium, may be used for preparation of the biosorbent CGC I which efficiently removes copper ions from water.
Acknowledgment
This research was performed within the FYBICH project organized by Contipro Group s.r.o. The author thanks his supervisor Dzianis Smirnou for a long-term professional help and consultation of this work. Appreciations also belong to Pavel Ondrška from the Control Analytical Laboratory Contipro Pharma a.s. for executing the ICP-OES tests and to Ondřej Žídek from the Nanotechnology Device Development Group Contipro Pharma a.s. for providing the scanning electron microscope photographs.

Literature


